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Pseudomonas aeruginosa Induces Membrane Blebs in Epithelial Cells, Which Are Utilized as a Niche for Intracellular Replication and Motility▽†

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Pseudomonas aeruginosa **is known to invade epithelial cells during infection and in vitro. However, little is known of bacterial or epithelial factors modulating *P. aeruginosa* intracellular survival or replication after invasion, except that it requires a complete lipopolysaccharide core. In this study, real-time video microscopy revealed that invasive *P. aeruginosa* isolates induced the formation of membrane blebs in multiple epithelial cell types and that these were then exploited for intracellular replication and rapid real-time motility. Further studies revealed that the type three secretion system (T3SS) of *P. aeruginosa* was required for blebbing. Mutants lacking either the entire T3SS or specific T3SS components were instead localized to intracellular perinuclear vacuoles. Most T3SS mutants that trafficked to perinuclear vacuoles gradually lost intracellular viability, and vacuoles containing those bacteria were labeled by the late endosomal marker lysosome-associated marker protein 3 (LAMP-3). Interestingly, mutants deficient only in the T3SS translocon structure survived and replicated within the vacuoles that did not label with LAMP-3. Taken together, these data suggest two novel roles of the *P. aeruginosa* T3SS in enabling bacterial intracellular survival: translocon-dependent formation of membrane blebs, which form a host cell niche for bacterial growth and motility, and effector-dependent bacterial survival and replication within intracellular perinuclear vacuoles.**

Pseudomonas aeruginosa infection is a significant cause of human morbidity and mortality (8, 23, 27, 62, 63). Predisposing factors include contact lens wear (cornea), burn wounds (skin), and preexisting disease or intubation of the airways. Exposed tissue surfaces lined with epithelia are the most common targets of *P. aeruginosa*; epithelial surfaces of the eye (cornea), skin, and airways are each exposed to a daily barrage of potential pathogens, but each normally resists infection. Major gaps remain in our understanding of the mechanisms by which healthy epithelia defend against microbes and by which epithelial compromise predisposes to infection with *P. aeruginosa* and other pathogens.

The type three secretion system (T3SS) is important for *P. aeruginosa* virulence in corneal, skin, airway, and systemic infections (53, 61). This system delivers effector proteins into host cells upon contact by the combination of a needle apparatus (allows effector secretion directly from the bacterial cell) followed by a translocon pore (inserted into host membranes for the intracellular delivery of effectors). The T3SS and its complex regulation in bacteria have been described in detail in several reviews (11, 14, 59, 65, 68, 70). At present, there are four known effectors encoded by *P. aeruginosa* to varying degrees: ExoS (~72% of isolates), ExoT (100% of isolates), ExoU (~28% of isolates), and ExoY (~89% of isolates) (20). ExoS and ExoT possess both ADP-ribosyltransferase and GTPase-activating protein activities, which induce apoptosis of host cells and interfere with actin cytoskeleton function (5, 43). ExoU is a phospholipase which is acutely toxic to mammalian cells (21, 52). ExoY is an adenylate cyclase which can also interfere with the host cell cytoskeleton (13, 60, 64).

While in vitro research has focused mostly on deciphering the active domains of T3SS effectors and their host cell molecular targets (1, 5, 12, 30, 31, 33, 43, 57, 60), in vivo studies have concentrated largely on proving that effectors and their in vitro-defined active domains contribute to disease (42, 53, 61, 71). Molecular and cellular relationships between in vitro-defined activities and the in vivo roles of the T3SS are yet to be established, in particular as they relate to the sequence of events enabling bacterial survival and to the numerous cell types and conditions under which bacteria interact with the infected host.

We have previously shown that *P. aeruginosa* invades epithelial cells in vitro and in vivo (24, 25, 42). It has also been shown that invasion requires bacterial interaction with host cell targets such as lipid rafts (39, 66, 67), which are themselves associated with specific targets for *P. aeruginosa* invasion, for example, the cystic fibrosis transmembrane conductance regulator (38, 49) and asialo-GM1 (10). These

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TABLE 1. Bacterial strains and mutants used in this study

Strain	T3SS description	Source or reference
6294	Wild type, functional T3SS	24
PAK	Wild type, functional T3SS	Joanne Engel, UCSF
PAO1	Wild type, functional T3SS	Maria-Cristina Plotkowski, State University of Rio de Janeiro, Brazil
PAO1*pscC*::pCR2.1	T3SS needle mutant; cannot secrete or translocate known effectors	Maria-Cristina Plotkowski, State University of Rio de Janeiro, Brazil
PAO1Δ*exsA*::Ω	No T3SS	Dara Frank
PAO1Δ*popB*	T3SS translocon mutant; secretes but cannot translocate known effectors	Arne Rietsch, Case Western Reserve University
PAO1Δ*exoSexoTexoY*	Triple effector mutant; functional needle and translocon apparatus without known effectors	Arne Rietsch, Case Western Reserve University
PA103Δ*exoUexoT*::Tc	Double effector mutant; functional needle and translocon apparatus without known effectors	Dara Frank
PA103*pscJ*::Tn*5*	T3SS needle mutant; cannot secrete or translocate known effectors	Joanne Engel, UCSF

targets interact with bacterial invasion ligands, including lipopolysaccharide (LPS), flagellin, and pili (10, 22, 69). Invasion also involves numerous host cell intracellular signaling proteins, including Src-family tyrosine kinases (15, 17, 18, 34), calcium-calmodulin (17), MEK-ERK (19), Akt (36, 37), and the actin cytoskeleton (35, 43, 44, 57). However, little is known of the intracellular fate of *P. aeruginosa* after internalization or of the bacterial or host cell factors involved in intracellular survival/replication, with the exception that a complete LPS core is required (16, 69). Here we tested the hypothesis that intracellular survival and replication of invasive *P. aeruginosa* in epithelial cells also requires an intact T3SS.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* strains and mutants used are listed in Table 1. Bacterial inocula were prepared from overnight cultures grown on trypticase soy agar plates (supplemented with antibiotics when appropriate) at 37°C for 14 to 16 h before suspension in appropriate serum-free cell culture medium (e.g., KGM-2 for human corneal epithelial cells) without antibiotics to a spectrophotometer optical density of 0.1 at 650 nm ($\sim 1 \times 10^8$ CFU/ml). Some strains were transformed with a green fluorescent protein (GFP)-encoding plasmid by heat shock transformation and selected on antibiotic containing media prior to overnight growth for experiments. Inocula were diluted to either $\sim 1 \times 10^6$ or $\sim 1 \times 10^7$ CFU/ml for intracellular survival or microscopy assays, respectively. Bacterial concentrations were confirmed by initial viable counts from inocula.

Cell culture. Human telomerase-immortalized corneal epithelial cells (hTCEpi) were maintained in 75-mm vented flasks in serum-free KGM-2 medium (Lonza, Walkersville, MD) until confluent as previously described (48). A549 human alveolar epithelial cells were grown in 75-mm vented flasks in Ham's F-12 medium (Lonza, Walkersville MD) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin-amphotericin until confluent, as previously described (54). Culture medium was replaced every 2 days after washing cells with sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO). Approximately 2 days before each experiment, cells were seeded onto 22-mm glass coverslips placed in non-tissue culture-treated six-well plates (for microscopy) or 24-well tissue culture plates (for intracellular survival assays) and grown to ~80% confluence. All cells were incubated at 37°C with 5% CO_2 during routine culture and during experiments. Primary or immortalized rabbit corneal epithelial cells were maintained in culture as previously described (25).

Intracellular survival/replication assays. Extended antibiotic survival assays were used to measure intracellular viability of *P. aeruginosa* in either hTCEpi or A549 cells. Cultured cells were washed twice with sterile PBS (500 μl). Bacterial inocula were prepared as described earlier (diluted in KGM-2 for hTCEpi cells or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% L-glutamine for A549 cells), and then 500 μl was added to each well of a 24-well tissue culture plate. Following a 3-h incubation at 37°C, the bacterial inoculum was removed and epithelial cells were treated with tissue culture medium containing gentamicin (200 μg/ml) for 1 h at 37°C to kill extracellular bacteria (25). To determine intracellular survival, some samples were incubated in gentamicin-containing solution for an additional 4 h (a total of 5 h of gentamicin treatment). Inoculation of samples was timed so that gentamicin treatments concluded simultaneously, to minimize sample disruption. To quantify intracellular bacteria, the gentamicin solution was removed, cells were washed twice with sterile PBS (500 μl), and then lysed with Triton X-100 (0.25% [vol/vol]) in PBS (15 min) (25). Viable intracellular bacteria in cell lysates were quantified by viable counts using MacConkey agar (PML Microbiologicals, OR). The difference in the number of viable bacteria recovered after 1 h of gentamicin treatment (invasion assay, 4-h time point) and 5 h of gentamicin treatment (survival/replication assay, 8-h time point) allowed determination of the fate of internalized bacteria over a 4-h interval. Time intervals were chosen to allow sufficient bacterial invasion and then intracellular residence time to detect differences in survival, replication, and bleb formation between wild-type and mutant bacteria. At least three samples were used for each group in each experiment, and experiments were repeated at least twice. Control experiments (not shown) confirmed that there were no significant differences in the growth rates of wild-type and mutant bacteria within cell culture media over an 8-h time period and that all strains were susceptible to killing by gentamicin or amikacin (experiments involving PA103*pscJ*::Tn*5*).

Exit assays. Modified antibiotic survival assays were used to measure host cell exit of *P. aeruginosa* from hTCEpi cells. Cultured cells were prepared and infected as described above for intracellular survival and replication experiments. Following a 3-h incubation at 37°C, bacteria were removed and epithelial cells were treated with tissue culture medium containing gentamicin (200 μg/ml) for 1 h at 37°C to kill extracellular bacteria (25). Gentamicin-containing solution was removed, and the cells were washed twice with sterile PBS (500 μl). For each group, some wells were then used to determine bacterial invasion as described above (4-h time point). To determine bacterial exit, other wells were treated with KGM-2 medium without antibiotics for an additional 1 h at 37°C to allow viable exit. Exiting bacteria were then quantified by viable count from cellular supernatants using MacConkey agar (5-h time point) and are expressed as a percentage of the number of original invading bacteria. At least three wells were used for each group in each of two experiments.

Microscopy. Experiments visualized by live video phase-contrast microscopy were performed using the same experimental protocol described earlier for extended antibiotic survival assays, except that glass coverslips of cultured epithelial cells (contained within six-well tissue culture plates) were inoculated with 2 ml of bacterial inocula at $\sim 1 \times 10^7$ CFU/ml for 3 h prior to gentamicin treatment. After 1 h or 5 h of gentamicin treatment (4-h and 8-h time points), a coverslip was removed from the tissue culture well, washed once with PBS (1 ml), and then placed into an Attofluor cell chamber (Molecular Probes) and maintained at 37°C in fresh gentamicin solution (200 μg/ml). Phase-contrast video microscopy was performed at 1,000× magnification, wherein bacterial interactions with host cells were captured as real-time movies and as still images for subsequent analysis.

For immunofluorescence microscopy, experiments were carried out as above but using bacteria expressing GFP for visualization (7). Infected and sham-inoculated cells were fixed at the indicated time intervals with 4%

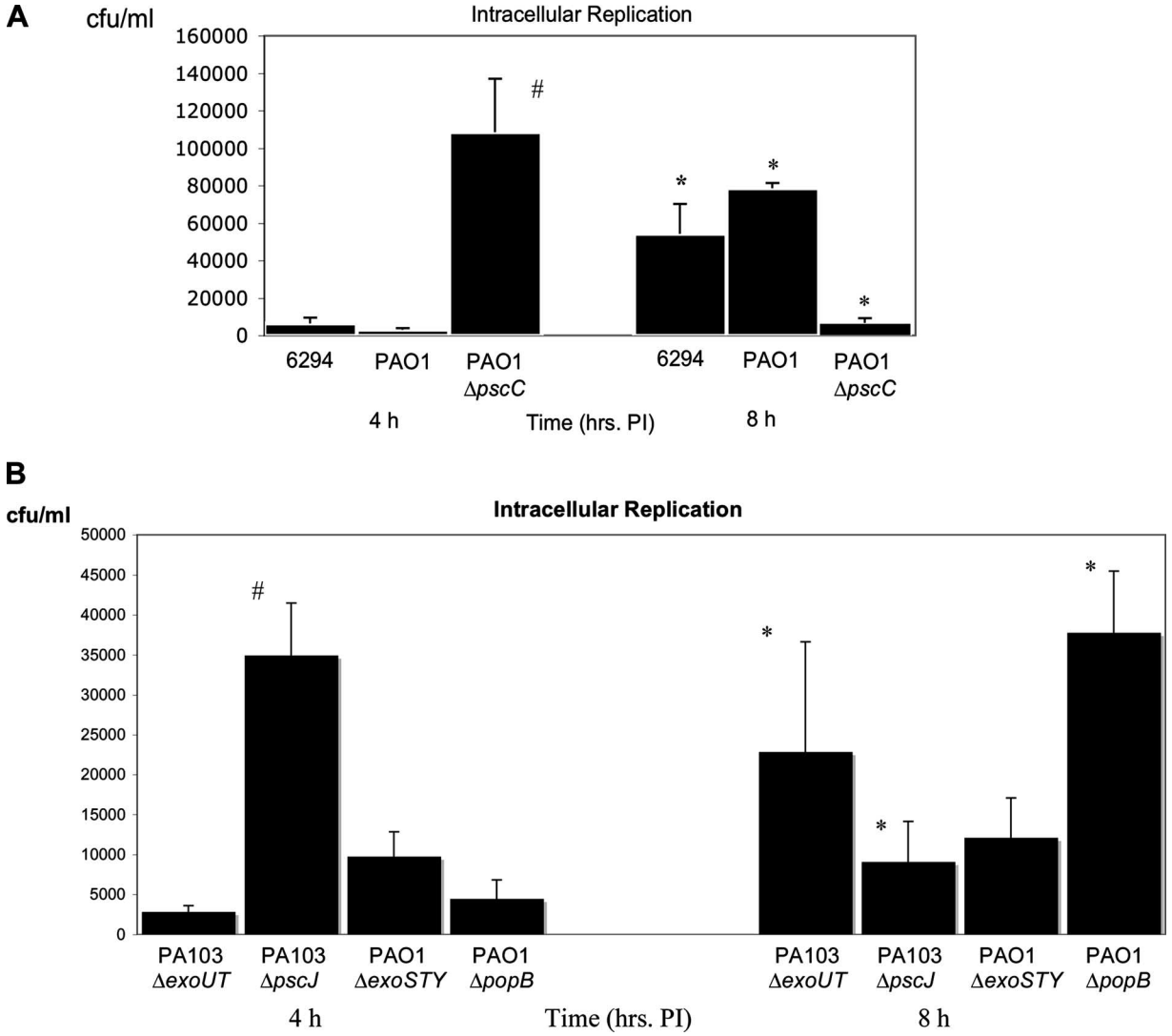

FIG. 1. A. Intracellular survival and replication of wild-type *P. aeruginosa* strains 6294, PAO1, and PAO1*pscC*::pCR2.1 (T3SS needle mutant) within human corneal epithelial cells after a 3-h incubation with 5×10^5 CFU of bacteria prior to 1-h or 5-h gentamicin treatments (4-h and 8-h time points, respectively). Only wild-type strains survived and replicated. B. A similar experiment comparing strains PA103Δ*exoUexoT*::Tc and PA103*pscJ*::Tn*5* (inoculum of 5×10^6 CFU for PA103 mutants) or PAO1Δ*exoSexoTexoY* and PAO1Δ*popB* (inoculum of 5×10^5). *, $P < 0.05$ compared to 4-h time point for each strain; #, $P < 0.05$ compared to PAO1 parent strain (A) or PA103Δ*exoUexoT*::Tc isogenic mutant strain (B).

paraformaldehyde (Sigma, St. Louis, MO) and washed with three exchanges of sterile PBS (10 min each), followed by cell permeabilization (0.1% [vol/vol] Triton X-100 in PBS; 10 min). After three additional PBS washes (10 min each), samples were blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) for 1 h at room temperature (or overnight at 4°C). Lysosome-associated marker protein 3 (LAMP-3) was labeled using mouse monoclonal anti-LAMP-3 primary antibody conjugated to rhodamine (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature (or overnight at 4°C). After three more washes with sterile PBS, samples were allowed to air dry and then mounted onto glass slides with Vectashield mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Fluorescent micrographs were captured by laser confocal scanning microscopy at 1,000× magnification (Zeiss).

Statistical analysis. Intracellular survival data are presented as the mean ± standard deviation (SD) for each time point. Differences between samples were evaluated for statistical significance using an analysis of variance (with the Fisher protected least significant difference used for post hoc analysis) with data containing more than two groups. When comparing two groups, Student's *t* test was used. *P* values of <0.05 were considered significant.

RESULTS

Mutations in the T3SS modulate *P. aeruginosa* intracellular survival and replication. Extended antibiotic survival assays were used to compare intracellular survival and replication of wild-type *P. aeruginosa* strains with that of various T3SS mutants (Table 1; Fig. 1). As expected, wild-type strains (PA01 and 6294) survived and replicated within corneal epithelial cells after they invaded (Fig. 1A). In contrast, a T3SS needle mutant (PA01*pscC*::pCR2.1) showed decreased intracellular viability despite higher levels of initial invasion (which was expected, since some effectors of the T3SS have antiphagocytic activity [12, 31]) (Fig. 1A). Similar results were found for a T3SS needle mutant in a different strain (PA103*pscJ*::Tn*5*) (Fig. 1B). In the same experiment, a mutant of PA103 (PA103Δ*exoUexoT*::Tc) that encodes no known effectors and a

translocon mutant of strain PA01 (PA01Δ*popB*) showed similar survival and replication patterns as wild-type bacteria (Fig. 1B). Interestingly, a PAO1 mutant that encodes no known effectors (PA01Δ*exoSexoTexoY*) lacked the capacity to replicate intracellularly (Fig. 1B).

One possible explanation for why needle mutants were found in reduced numbers at later time points is that they were able to kill epithelial cells and therefore escape from them. However, cell lysis was not observed for cells infected with any of the T3SS mutants (including both PAO1 and PA103 strains). Furthermore, exit assays revealed that needle mutants were actually deficient in their capacity to exit cells (e.g., 0.006% of PA01*pscC*::pCR2.1 needle mutants that had invaded cells exited versus 38.9% of PAO1 wild type). Together, the results of intracellular survival assays and exit assays suggested that needle mutants lacked the capacity to maintain their viability within epithelial cells.

The T3SS affects the intracellular location of *P. aeruginosa*. Phase-contrast live video microscopy was used to compare the intracellular location of wild-type *P. aeruginosa* and the various T3SS mutants 4 and 8 h postinfection (Table 1; Fig. 2 and 3). Wild-type strain PA01 was located within large plasma membrane blebs that had formed on corneal epithelial cells at both 4 and 8 h (Fig. 2A and B, respectively). The blebs appeared translucent, enlarged with time, and contained numerous bacteria which were seen replicating and demonstrating rapid real-time motility within. Both the transparency of the blebs and the rate of bacterial motility within suggested that they lacked the cytoskeletal structure normally found within the cytoplasm of cells. Thus, they likely represent areas where the plasma membrane is no longer attached to the actin cortex (see Videos S1 to S5 in the supplemental material). Motility and replication occurred even as the cells were incubated in the non-cell-permeable antibiotic gentamicin, which kills extracellular bacteria. These results confirmed that the bacteria were "inside" the cell and also that the plasma membrane had not become permeabilized, which would have allowed the antibiotic to also kill intracellular bacteria. Occasionally, blebs containing bacteria were observed to detach from an infected epithelial cell and then to roll or float away to other locations (see Video S6 in the supplemental material). Control uninoculated cells maintained normal morphology and viability throughout these experiments (Fig. 2C and D).

Different results were found for the T3SS mutants. For example, the needle mutant of strain PA01 (PAO1*pscC*::pCR2.1) did not form membrane blebs but instead appeared to be localized to small perinuclear vacuoles at both the 4- and 8-h time points (Fig. 2E and F, respectively). Both the effector mutant (PA01Δ*exoSexoTexoY*) and translocon mutant (PA01Δ*popB*) were also found to locate within perinuclear vacuoles at 8 h postinfection, but they also lacked the capacity to form membrane blebs (Fig. 2H and J, respectively). Differences were found between the effector mutant and the translocon mutant at 4 h postinfection. The translocon mutant was already found located in perinuclear vacuoles (Fig. 2I), while the triple effector mutant was not located in either perinuclear vacuoles or membrane blebs (Fig. 2G). Cell lysis was not observed for cells infected with these T3SS mutants that lacked blebbing capacity. Cells containing these bacteria within perinuclear vacuoles appeared otherwise healthy morphologically.

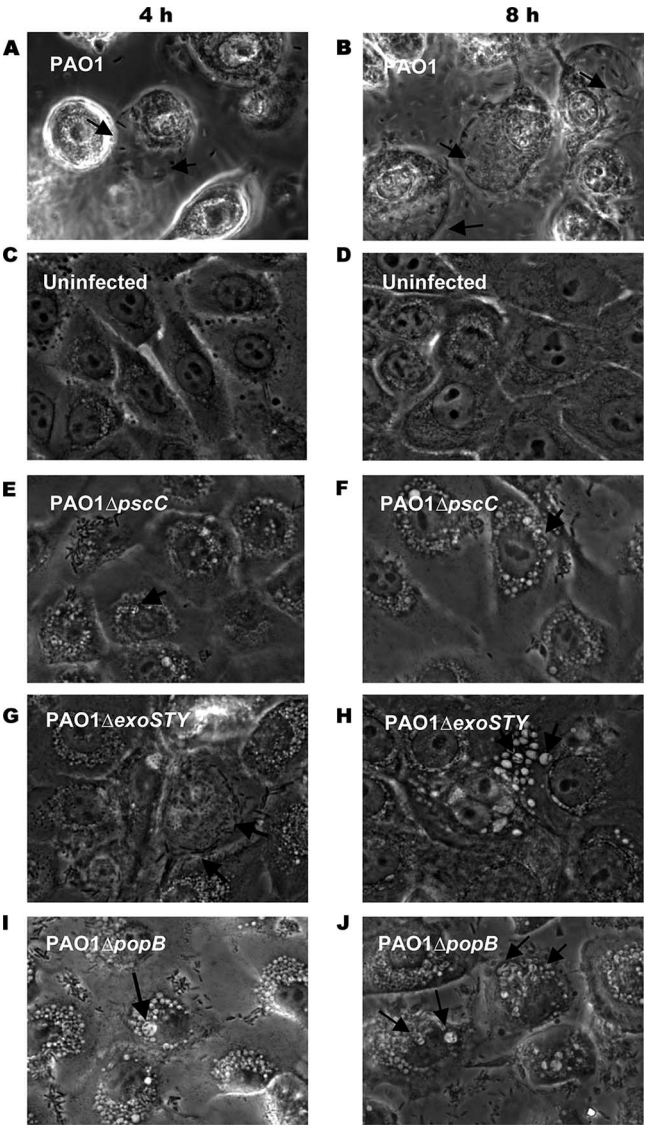

FIG. 2. Intracellular location of *P. aeruginosa* wild-type PAO1 and T3SS mutants within human corneal epithelial cells after a 3-h incubation with 2×10^7 CFU of bacteria followed by 1-h or 5-h gentamicin treatment (4-h and 8-h time points, respectively). PAO1 occupied spacious membrane blebs at 4 h (A) and 8 h (B). Uninfected cells maintained a healthy state at each time point (C and D, 4 and 8 h, respectively). A T3SS needle mutant, PAO1*pscC*::pCR2.1, was found in perinuclear vacuoles at 4 h (E) and 8 h (F). The mutant PAO1Δ*exoSexoTexoY* was not found in blebs or vacuoles at 4 h (G) but was found in vacuoles at 8 h (H). The translocon mutant, PAO1Δ*popB*, also localized to vacuoles at each time point (I and J, 4 and 8 h, respectively). See also Video S1 in the supplemental material for real-time microscopy of wild-type strain PAO1 swimming inside blebs in this cell line.

T3SS mutants of the cytotoxic strain PA103 have previously been shown to adopt an invasive phenotype (31). Thus, the intracellular location of the effector mutant of PA103 (PA103Δ*exoUexoT*::Tc) was compared to that of a T3SS needle mutant in the same strain (PA103*pscJ*::Tn*5*). Similar to the

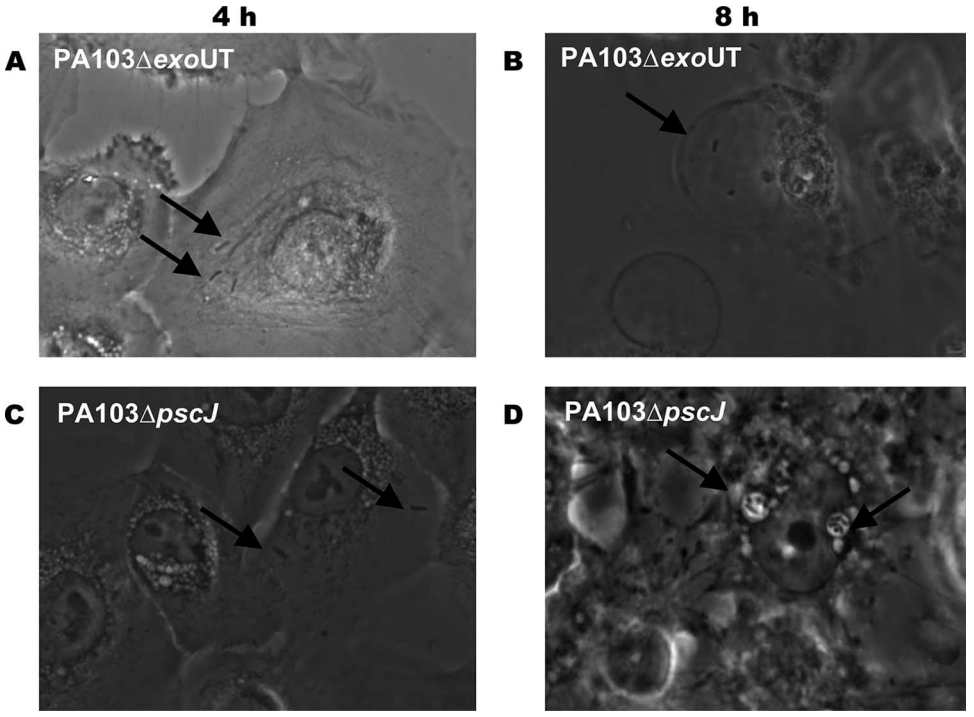

FIG. 3. Intracellular location of T3SS mutants of *P. aeruginosa* strain PA103 after a 3-h incubation with human corneal epithelial cells (2×10^7 CFU of bacteria) followed by 1-h or 5-h gentamicin treatment (4-h and 8-h time points, respectively). The double effector mutant PA103Δ*exoUexoT*::Tc was not seen in membrane blebs or vacuoles at 4 h (A) but occupied membrane blebs at 8 h (B). The needle mutant PA103*pscJ*::Tn*5* was also not observed within perinuclear vacuoles at 4 h (C) but was located within these vacuoles at 8 h (D). See also Video S2 in the supplemental material for real-time microscopy of strain PA103Δ*exoUexoT*::Tc inside blebs in this cell line.

results found with strain PAO1, neither mutant appeared to be located in membrane blebs or vacuoles 4 h postinfection (Fig. 3A and C, respectively). Also in accordance with results obtained with strain PAO1, the needle mutant was found within perinuclear vacuoles by 8 h postinfection (Fig. 3D). However, in contrast to PAO1, the double effector mutant of strain PA103 was found to retain the capacity to form and traffic to large, translucent membrane blebs similar to those induced by wild-type PAO1 (Fig. 3B; see also Video S2 in the supplemental material).

Wild-type *P. aeruginosa* also induced membrane blebbing in other epithelial cell types (Fig. 4). These included primary cultured rabbit corneal epithelial cells after infection with wild-type strain 6294 (Fig. 4A and B; see also Video S3 in the supplemental material) and simian virus 40-transformed rabbit corneal epithelial cells after infection with wild-type PAK (Fig. 4C and D; see also Video S4 in the supplemental material). For human alveolar epithelial cells (A549 cells), wild-type PAO1 was not seen within membrane blebs at 4 h (Fig. 4E) but did induce membrane blebs at 8 h postinfection (Fig. 4F; see also Video S5 in the supplemental material).

Intracellular colocalization of *P. aeruginosa* and T3SS mutants with the late endosomal/lysosomal marker LAMP-3. Immunofluorescence microscopy was used to investigate the colocalization of intracellular *P. aeruginosa* and the T3SS mutants with the late endosomal/lysosomal marker LAMP-3 (Fig. 5). Uninfected human corneal epithelial cells showed dense perinuclear labeling of LAMP-3 (Fig. 5A). Cells infected with wild-type PAO1 (GFP labeled) showed a much stronger LAMP-3 signal which was diffusely distributed in the cytoplasm rather than localized around the nucleus. Wild-type bacteria did not colocalize with LAMP-3 (Fig. 5B). Indeed, LAMP-3 and bacterial location were found to be mostly mutually exclusive. Conversely, GFP-labeled T3SS mutants of strain PA103 (PA103Δ*exsA*) were found colocalized with LAMP-3, and LAMP-3 distribution was found perinuclear in a similar distribution to uninfected cells (Fig. 5C). Control experiments confirmed that PAO1Δ*exsA* mutants were also reduced in their capacity for intracellular survival/replication (Table 2). Slight differences compared to *pscC* or *pscJ* mutants suggest that this regulatory component of the T3SS impacts other factors also involved in intracellular behavior or that these mutants which lack the capacity to express all T3SS components have a different physiology than mutants capable of making all but one of the T3SS-related components (i.e., *pscC* or *pscJ* mutants).

Finally, the translocon (PAO1Δ*popB*) mutant, which we had shown retains the capacity to replicate despite localizing to perinuclear vacuoles, did not colocalize with LAMP-3, which remained perinuclear but appeared mostly in regions away from where bacteria were and appeared less intense than in uninfected cells (Fig. 5D).

DISCUSSION

We have previously shown that *P. aeruginosa* can invade epithelial cells (24, 25) and can replicate inside cells after invasion, requiring a complete LPS core (16). The data presented in this study show that the survival and replication of *P.*

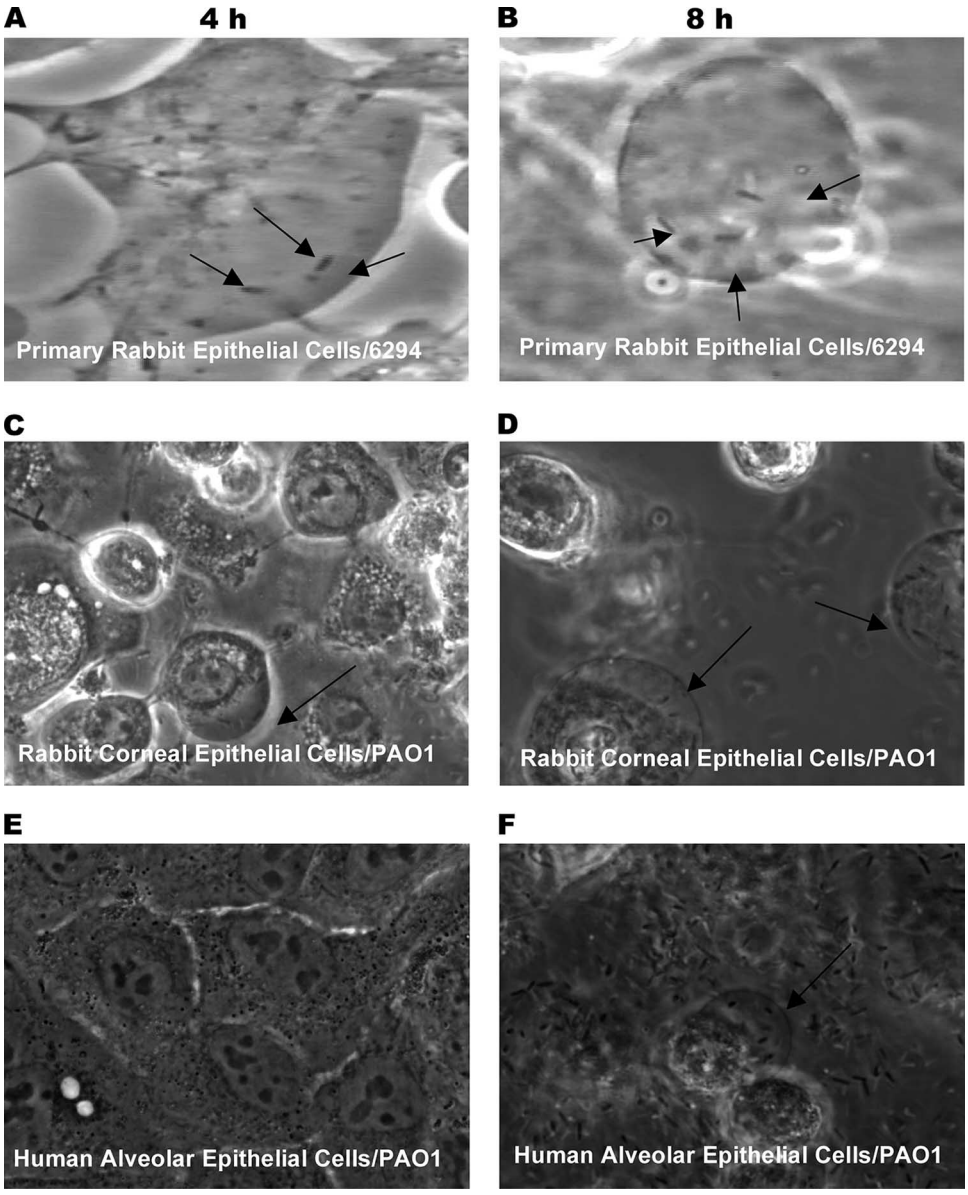

FIG. 4. *P. aeruginosa*-induced membrane blebbing in other epithelial cell types. (A and B) Primary cultured rabbit corneal epithelial cells infected with 2×10^6 CFU of clinical ocular isolate strain 6294 for 3 h prior to gentamicin treatment. Images were taken between 4 and 8 h postinfection. (C and D) Simian virus 40-immortalized rabbit corneal epithelial cells infected with 2×10^7 CFU of strain PAK for 3 h prior to gentamicin treatment were then observed at the 4-h (C) and 8-h (D) time points. Human airway (alveolar) epithelial cells (A549) infected with 2×10^7 CFU of strain PAO1 for 3 h prior to gentamicin treatment were then observed at 4 h (E) and 8 h (F). See also Videos S3 (6294), S4 (PAK), and S5 (PAO1) in the supplemental material for real-time microscopy of bacteria swimming inside blebs corresponding to the still images.

aeruginosa within corneal and other epithelial cells are modulated by the ExsA-regulated T3SS. Defects in the T3SS that prevent the secretion of effectors or other components (needle mutants) were found to result in the loss of intracellular viability of bacteria after invasion associated with bacterial localization within perinuclear vacuoles and colocalization with the late endosomal/lysosomal marker LAMP-3. In contrast, in the presence of a functional T3SS, wild-type *P. aeruginosa* was found to localize to membrane blebs, a novel survival niche wherein it demonstrated the capacity for intracellular replication and motility and a lack of LAMP-3 colocalization.

Data collected using other T3SS mutants of *P. aeruginosa* strain PAO1 (i.e., for comparison between effector and translocon mutants) showed that the capacity to secrete effectors was required to enable replication of bacteria within perinuclear vacuoles. However, effector translocation to the host cell cytoplasm may not be required for survival in perinuclear vacuoles, since a translocon mutant (which retains the capacity to secrete effectors) survived and replicated within these vacuoles without inducing bleb formation.

Several different types of plasma membrane blebbing have been previously reported. For example, blebbing can occur as a result of mechanical perturbation of cells or normal cellular processes, such as apoptosis or cytokinesis (9). Interestingly, *P.*

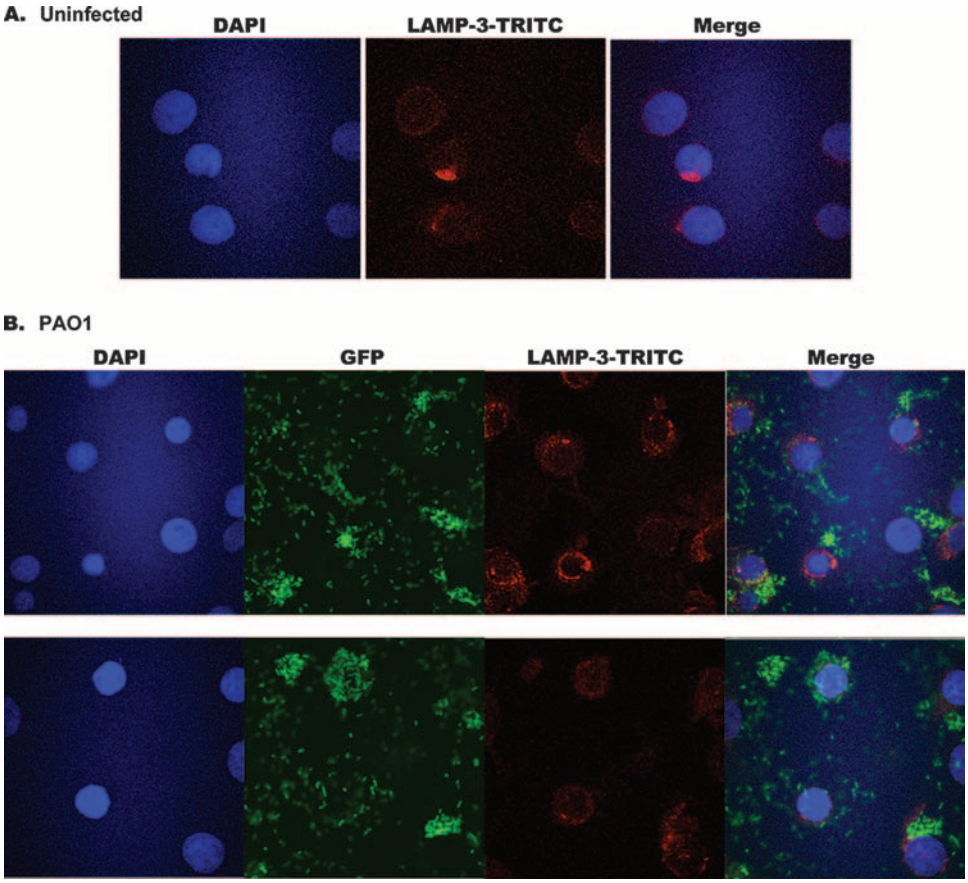

FIG. 5. Intracellular colocalization of *P. aeruginosa* with the late endosome/lysosomal marker LAMP-3. Uninfected human corneal epithelial cells labeled with LAMP-3 (red) and DAPI (blue) (A). Wild-type PAO1 bacteria expressing GFP (green) were largely unassociated with LAMP-3 (B). The T3SS-null mutant PAO1Δ*exsA* expressing GFP was associated with cells in fewer numbers and colocalized with LAMP-3 adjacent to the nucleus (C). The T3SS translocon mutant PAO1Δ*popB* was seen unassociated with LAMP-3 (D). All images were captured at 8 h postinfection with laser scanning confocal microscopy (see Materials and Methods). In each instance, cells were infected with 2×10^7 CFU bacteria for 3 h before gentamicin treatment.

aeruginosa has been reported to induce apoptosis following delivery of toxins (ExoS and exotoxin A) to host cells (30, 33). As physiological blebs are relatively small and retract rapidly (9), the continuous expansion of the *P. aeruginosa*-infected blebs and the replication/motility of bacteria in the presence of gentamicin-containing culture medium suggest a process actively driven by the intracellular bacteria. Interestingly, physiological bleb retraction has been shown to require RhoA, a known host cell target of *P. aeruginosa* T3SS effectors, e.g., ExoS and ExoT (1, 5, 57).

Also of possible relevance is that *P. aeruginosa* (strain PAO1) has been shown to occupy intracellular pods within cultured airway epithelial cells, and during this occupation the bacteria up-regulated expression of OprF, a biofilm-associated outer membrane protein and showed increased resistance to intracellular antimicrobials (26). The derivation and composition of the *Pseudomonas*-induced blebs observed in the present study are unclear, and it is possible that they share characteristics with one or more of these previously observed phenomena (e.g., toxin-induced blebs or intracellular pods).

Intracellular motility of *P. aeruginosa* within blebs was rapid, i.e., visible in real time (see the videos in the supplemental material), while actin-based motility is known to be extremely slow (detectable only using time-lapse video recording) (55). Recent studies of physiological membrane blebs have shown expanding blebs to be devoid of actin during detachment from the cortex (9). The speed and appearance of *P. aeruginosa* intracellular motility suggested flagellum-mediated swimming and that membrane blebs were devoid of dense cytoskeletal structure. Further implicating flagellum-mediated swimming motility are our results for strain PA103. This strain was found to be nonmotile within blebs (see Video S2 in the supplemental material); PA103 is known to lack flagella and therefore the capacity for swimming motility.

The PAO1Δ*popB* (translocon) mutants lacked the capacity to cause blebbing, although they demonstrated efficient intracellular survival and replication. The mechanism for promoting survival of these mutants within perinuclear vacuoles is not known, but it might involve the fact that these mutants remain able to secrete effectors despite their lack of ability to translocate them across host cell membranes. A role for effectors is further suggested by the fact that effector mutants which also traffic to perinuclear vacuoles have a reduced capacity for replication. The absence of LAMP-3 from vacuoles containing

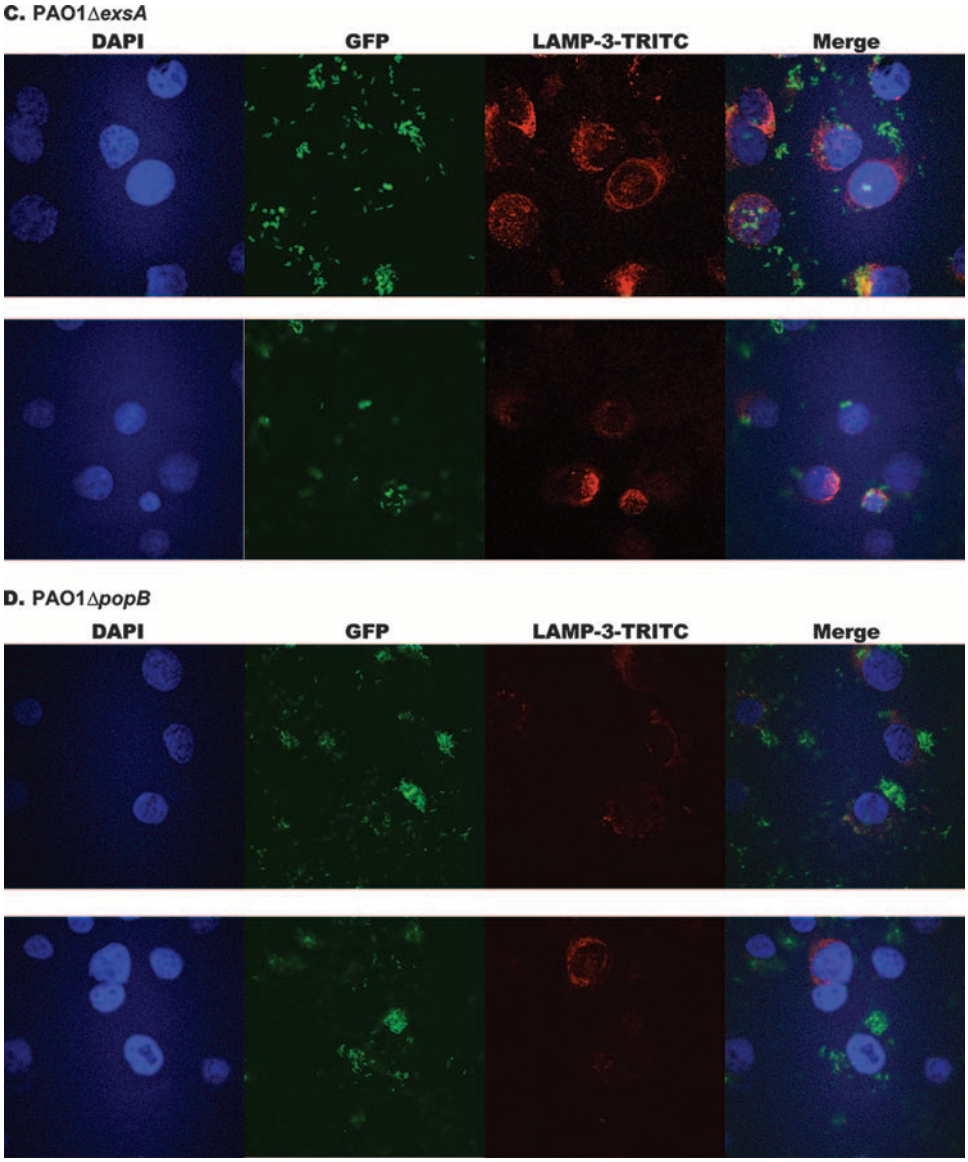

FIG. 5—*Continued*.

translocon mutants suggests that effectors might hinder vesicular maturation, which is usually required for the killing of intracellular pathogens by other mammalian cell types (3, 6, 45, 47). *Salmonella* spp. are known to use their T3SS toxins to prevent fusion of the *Salmonella*-containing vacuole with lysosomal compartments (32, 46). The reduction of intracellular survival/replication observed with the triple effector mutant (PAO1Δ*exoSTY*) of PAO1 in comparison with the PAO1Δ*popB* mutant suggests a role(s) for one or more of these effectors in *P. aeruginosa* intracellular survival in perinuclear vacuoles. One possible scenario for our findings is that without secretion of T3SS effectors, *P. aeruginosa* is ultimately killed by trafficking to phagolysosomes within epithelial cells and that one or more effectors prevent normal phagolysosome maturation/function by one or more mechanisms, e.g., destabilization of endosome trafficking, prevention of lysosomal fusion, or the inactivation of lysosomal killing mechanisms.

TABLE 2. Intracellular viability of an *exsA* mutant of *P. aeruginosa* strain PAO1

P. aeruginosa strain	Mean ± SD intracellular bacteria (CFU/ml)		*P* value for 4 h vs 8 h (*t* test)
	4 h postinfection	8 h postinfection	
PAO1	36,556 ± 7,183	118,556 ± 15,959	0.0013
PAO1*exsA*::Ω	34,222 ± 3,791	34,778 ± 7,381	0.9133

It is of interest that the effector mutant of cytotoxic strain PA103 used in our experiments, which lacks all known effectors (PA103Δ*exoUexoT*::Tc), remained capable of intracellular survival and replication and that it also localized to membrane blebs. This was especially intriguing since the needle mutant of this strain lacked all of these capacities, suggesting that there are unknown T3SS-related factors in PA103 (which may pos-

sess similar activities as ExoS and ExoT) that contribute to intracellular pathogenesis, as previously suggested by others (40, 41). Alternatively, it remains possible that the needle and/or translocon structures participate in intracellular survival/replication or bleb formation for this strain.

While our data support a role of *P. aeruginosa* T3SS in epithelial membrane bleb formation (effector, needle, and translocon mutants of PAO1 all lacked this capacity), it is not clear whether this role is direct or indirect. It is possible that the role of the T3SS is in steering bacteria away from a different fate, i.e., host cell-driven trafficking to perinuclear vacuoles. However, the T3SS could conceivably play direct roles in bleb formation. For example, the T3SS translocon pore may provide the stimulus for blebbing, or T3SS effectors could contribute by manipulating the cytoskeleton and its connection to the cell membrane.

The unique membrane blebs induced by invasive *P. aeruginosa* appear to contrast sharply with the behavior of other intracellular pathogens, e.g., *Shigella* spp., *Listeria* spp., and *Rickettsia* spp. (escaping from endosome, actin-based intracellular motility, or cell-cell spread) (2), *Salmonella* spp. (blocking endosome-lysosome fusion) (32, 45, 56), or *Brucella* spp. (escaping the endosomal pathway to replicate inside other organelles, e.g., endoplasmic reticulum-derived compartments) (46). *Brucella* and *Burkholderia* evasion of lysosomes and *Coxiella* survival within them also occurs, although those mechanisms are poorly understood (4, 28, 46, 51). In each instance, these pathogens achieve a goal of intracellular survival, replication, and dispersal while being protected from intracellular and extracellular components of innate and acquired immunity. Our data suggest that these membrane blebs may provide a similar function for *P. aeruginosa* in that they could facilitate bacterial persistence within epithelial barriers of the human body. Although *P. aeruginosa* did not appear to utilize direct intracellular cell-to-cell spread (adjacent uninfected cells did not become infected after the antibiotic was added), our observation that infected blebs could detach from cells in vitro also suggests a possible mechanism for bacterial dispersal to other cells or tissues (see Video S6 in the supplemental material).

Our data suggest that the T3SS allows *P. aeruginosa* to evade lysosomal killing within corneal epithelial cells after invasion. While it is known that epithelial cells can have lysosomal activity (29, 50, 56, 58), bacterial invasion of these cells is generally assumed to be a bacterium-driven pathogenic strategy. The loss of viability of T3SS needle mutants, and also their location within LAMP-3-positive, perinuclear vacuoles, suggests that corneal epithelial cells may actively degrade invading pathogens as a novel innate defense. Since the cornea is immune privileged, this epithelial defense may help prevent infection (if it occurs in vivo) and contribute to maintenance of a healthy tissue at this and similar epithelial surfaces that are continuously exposed to the environment and only infiltrated by phagocytes during inflammation. The observation that *P. aeruginosa* can avoid this fate by surviving/replicating within intracellular vacuoles, or in membrane blebs from which they may readily escape to the extracellular environment, may contribute to its success as an opportunistic pathogen.

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